Natural Antioxidants Present In Capsicum Annum (Bell Pepper) Extracts Modulate Calcineurin Activity Under Conditions Of Oxidative Stress

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Abstract: The main objective of the study is to investigate the effect of natural antioxidants like carotenoids present in vegetables on the activity of the calcium/calmodulin stimulated protein phosphatase, Calcineurin. The activity of the calcium /calmodulin regulated protein phosphatase, Calcineurin was assayed in the bovine brain homogenate in presence and absence of natural antioxidants isolated from capsicum annum (bell pepper) extracts. The tissue homogenates were then incubated with hydrogen peroxide followed by incubation with natural antioxidants in bell pepper extracts. Calcineurin activity was inhibited on treatment with hydrogen peroxide which generates peroxyl radical. Synthetic antioxidants (BHT and BHA) inhibited calcineurin activity to an extent of greater than 85% of the control activity. Natural antioxidants like carotenoids extracted from bell peppers could enhance the Calcineurin activity in vitro. The Calcineurin activity was also restored to normal in extracts treated with hydrogen peroxide followed by incubation with bell pepper extracts. The results obtained above demonstrate that antioxidants present in bell pepper extracts did not inhibit Calcineurin activity in contrast to synthetic antioxidants which inhibited the activity of Calcineurin. Substituting synthetic antioxidants with natural antioxidants in packaged foods could serve as inexpensive and a healthy alternative for preserving packaged foods.

Keywords: Natural antioxidants, Calcineurin

1.Introduction.

Antioxidants are molecules that have the ability to reduce free radical damage and prevent oxidation. Synthetic antioxidants such as Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are added as food preservatives in packaged food. From the research, it is clear that there is an associated link between BHT, BHA and instigation and promotion of tumor formation [7]. These phenolic antioxidants scavenge a variety of free radicals and influence the activities and/or expression of several signal transducing molecules including mitogen activated protein. Another important signaling molecule that is affected by BHT and BHA is Calcineurin, a calcium-calmodulin dependent protein phosphatase [5]. Defective Calcineurin signaling has been proved in the manifestation of several clinical conditions including cancer, metabolic aberrations and degenerative brain diseases [3]. Strengthening of cellular defense mechanism by administering phytoneutrients provides an important strategy for disease management [1]. The important phytoneutrients that have antioxidant property are carotenoids and flavonoids. Among the vegetables, Capsicum annum (bell pepper) is a good source of these antioxidants [6]. Thus, the main aim of this study was to study the effect of these natural antioxidants in bell peppers on the Calcineurin activity.


Preparation of pepper extract.

Fruits of Capsicum annum (bell pepper) were cleared of the seeds and weighed accurately. 100gms of the bell pepper was blended in a kitchen scaled food processor and centrifuged at 8000 rpm to remove the debris. The supernatant was then boiled for 20 minutes and filtered through Whatmann 4 to remove the particulate matter. The supernatant was then boiled for 20 minutes and filtered through Whatmann 4 to remove the particulate matter.

Extraction of total carotenoids

The bell peppers (0.5g) were homogenized and saponified with 2.5ml of 12% alcoholic potassium hydroxide in a water bath at 60° C for 30 minutes. The saponified extract was transferred to a separating funnel containing 10-15ml of petroleum ether and mixed well. The lower aqueous layer was then transferred to another separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colourless. A small amount of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture.
Preparation of calcineurin extract from sheep brain:

Crude Calcineurin extracts were prepared from sheep brain. All the steps were carried out at 4°C. Essentially, the tissue was homogenized in buffer A (20mM Tris (pH7.2) and 1mM CaCl₂) and centrifuged at 4000 rpm to remove the debris. The supernatant was further centrifuged at 12,000rpm for 10 min. The supernatant was subjected to ammonium sulphate precipitation to precipitate the total protein. The protein precipitate was then suspended in buffer B (10mM Tris pH 7.2) and dialyzed against the same buffer.

Calcineurin assay.

Calcineurin activity was assayed in a total volume of 50 μl containing 25 μl of 2X assay buffer (200 mM NaCl, 100 mM Tris [pH 7.5], 12 mM MgCl₂, 1 mM CaCl₂) and 5 μl (∼15μg protein) of the protein dialysate [4]. The homogenates were incubated with 2X-assay buffer for 10 min at 30°C. Subsequently, incubation with the carotenoid fraction of the bell pepper extract (5µl) was carried out for 10 min at 30°C in the presence and absence of 1 mM hydrogen peroxide (H₂O₂). The total volume was adjusted to 50 μl by the addition of water. Reactions were initiated by adding RII phosphopeptide [5 μM] and incubated for a further period of 10 min at 30°C. Reactions were terminated by addition of 100 μl of Malachite green reagent (3 vol. of 0.045% Malachite green and 1 vol. of 4.2% ammonium molybdate in 4N HCl). The color was allowed to develop for 30 min and the absorbance read at 660 nm. The calcineurin activity was calculated as nmoles of inorganic phosphate released/min.

3.RESULTS.

Standard Calcineurin (15nM) was assayed for its phosphatase activity using RII phosphopeptide as the substrate. The activity was assayed in the presence of hydrogen peroxide and upon treatment with hydrogen peroxide and pepper extracts. The results obtained are depicted in fig1. The results demonstrated an inhibition of calcineurin activity on being treated with hydrogen peroxide which generates peroxyl radical. Calcineurin activity was restored upon treatment with pepper extracts.

The results obtained above indicated modulation of the activity of standard calcineurin by pepper extracts. Hence, it was of interest to compare the effect of synthetic antioxidants like butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA) with that of natural antioxidants present in pepper extracts. All further studies were carried out on crude brain preparations. The brain dialysates containing crude calcineurin, were treated with synthetic antioxidants (BHT, BHA and Trolox) and the results obtained are depicted in fig 2.
demonstrated an increase in Calcineurin activity (fig 3).

![Calcineurin activity in presence of natural antioxidants](image)

**Fig. 3.** Calcineurin activity in presence of natural antioxidants. The calcineurin activity was assayed in the presence of pepper extract and carotenoid extract which are rich sources of natural antioxidants.

![Calcineurin activity after treatment with hydrogen peroxide](image)

**Fig. 4:** Calcineurin activity after treatment with hydrogen peroxide. The dialysates were treated with hydrogen peroxide (control). The dialysates were then incubated with pepper extract as well as carotenoid extracts. Subsequently, Calcineurin activity was determined. The values depicted are average of 4 different experiments done in triplicate. The data were analysed by unpaired t-test and found to be statistically significant with a p value =0.032

The brain homogenates were then sequentially treated with hydrogen peroxide, an important source of peroxyl radical followed by treatment with the pepper extracts and carotenoid extracts respectively to study the role of natural antioxidants in pepper extracts. The results obtained are depicted in figure. 4. The oxidative effect of hydrogen peroxide was evident by a decrease in Calcineurin activity. Incubation with hydrogen peroxide followed by treatment with pepper extracts as well as carotenoid extract restored the Calcineurin activity to normal.

4. Discussion

Calcineurin, also known as protein phosphatase 2B, plays a vital role in several intracellular signalling events. It is the only calcium dependent phosphatase which serves as an important transducer of calcium signals. The Fe\(^{3+}/Zn^{2+}\) bimetallic catalytic centre of Calcineurin makes it a potential target for oxidative stress. In view of its extreme sensitivity to deactivation by reactive oxygen species, Calcineurin closely interacts with superoxide dismutase which protects it against oxidation and the concomitant loss of metal ions at the active site. In view of the wide spread use of synthetic antioxidants like butylated hydroxyl anisole(BHA) and butylated hydroxyl toluene(BHT) as food preservatives, it was of interest to study and compare the effect of synthetic antioxidants and natural antioxidants like carotenoids on commercially available bovine brain calcineurin as well as crude brain preparations.

Considerable evidence exists for the role of antioxidants in fruits and vegetables in the maintenance of health and in disease prevention. Thus, it was of interest to study the effect of natural antioxidants present in bell pepper extracts on Calcineurin activity. When the crude brain homogenate was incubated with whole pepper extracts as well as carotenoid extracts, it was observed that the Calcineurin activity was reduced by whole pepper extracts in comparison to carotenoid extract. This could be attributed to the inhibitory effect of flavonoids present in pepper extracts on Calcineurin activity. However, the carotenoid extracts enhanced the activity of calcineurin in brain homogenates. As the Calcineurin activity was enhanced by carotenoid extracts, it was of interest to study the antioxidant effect of these extracts on Calcineurin activity after treatment with hydrogen peroxide. The oxidative effect of hydrogen peroxide was evident by a decrease in Calcineurin activity. The results presented herein demonstrate that natural antioxidants like carotenoids help in restoring Calcineurin activity to normal, under conditions of oxidative stress. These results assume significance keeping in view the critical role of Calcineurin in health and disease. Further studies need to identify the effects of other similar antioxidants present in fruits and vegetables.
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6. References:


